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SMUFD D/A ltr, 14 Feb 1972

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AD836235

TRANSLATION NO. 1253

DATE: 11 Dec. 1964

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

BIOCHEMICAL STUDIES ON RICE BLAST DISEASE

Translation of an article by Hiroshi Otsuka, Kinjiro Tamari, and Nagahiro Ogasawara in the Japanese language journal, Journal of the Agricultural Chemical Society of Japan, Vol. 32, No. 11, 1958, pp. 890-893.

PART X

Biochemical Classification of Piricularia Oryzae Cavara. (5)

As a result of the tests we had conducted up to the time the previous report was made, on 45 strains of piricularia oryzae we were able to classify the latter into 11 types, according to whether they required biotin, possessed nitrate reducibility [literal], and according to the differences in the utilization of sodium nitrite, inulin, sorbose, and tryptophane. We found that there was a close relationship between our classification and the system developed by the National Institute of Agricultural Sciences. We conducted similar tests on 55 strains which were presented to us by the Nagano Agricultural Testing Station and obtained results identical to the previous tests (1).

As a result of testing culture filtrates and acid hydrolyzates of mycelia in order to determine whether strains No. 1 and No. 2 from the Faculty of Agriculture of Hokkaido University which do not require biotin, were capable of producing biotin, we found that such strains as No. 1 and No. 2 did not produce it.

From the above it can be assumed that the form of sugar metabolism in these strains differs from other strains. We considered the biochemical role of biotin (2), cultivated piricularia oryzae in culture media containing succinic acid, fumaric acid, and citric acid, and separately each of these with 1/500 M of malonic acid added, and, for purposes of contrast, in a medium employing malonic acid as a carbon source.

Inoue and Hayami (3) studied the relationship between the decomposability of cellulose and the pathogenicity of piricularia oryzae, and since research has recently been conducted on pectinase in plant pathogenes, we report here on our tests on the use of cellulose and pectinase in piricularia oryzae.

Tests.

(1) The total number of strains employed was 47, including the 45 strains tested in Part I of Report No. 10 on piricularia oryzae (4) and the two strains CO-homo and SO-homo received from professor Suzuki Hashio of the Faculty of Agriculture of the Tokyo University of Agriculture and Technology.

(2) Culture medium was the same as in Report No. 10 (Part I).

(3) Test on the ability of strains No. 1 and No. 2 to produce biotin. We made bioassays of the culture filtrates and acid hydrolysates of rinsed mycelia of strains No. 1 and No. 2, which were grown at 25° for 14 days in a biotin-free synthetic culture medium.

The culture filtrate and acid hydrolizates were heated and hydrolized for one hour at 15 pounds in $6NH_2SO_4$ according to L.D. Wright's method (5). The test substances were extracted with ether and the ether -- soluble portion excluded. The remainder was then neutralized in $Ba(OH)_2$ and subjected to quantitative analysis.

Biotin was estimated in the same manner as in the previous report. After cultivating Lactobacillus arabinosus ATCC 8014 in an identical basic culture medium, determination was carried out by the acid titration method using 1/20 N NaOH.

TABLE 1. BIOTIN CONTENT IN CULTURE FILTRATE AND IN BACTERIA

Strain	Biotin in culture filtrate (mγ/ml)	Biotin in bacteria (mγ/g)
No. 1	0.0	27.1
No. 2	0.0	71.4

(4). Test of utilization of organic salts. The basic culture medium has the same constituents as in Report 10 (Part II). We previously observed the growth of bacteria in testing the utilization of organic salts as a carbon source, and in this report we conducted tests in order to determine the quantitative relationship. We employed 2% respectively of sodium fumarate, sodium succinate, sodium citrate, and sodium malonate, and 1/100M of malonic acid added to the sodium fumarate, sodium succinate, and sodium citrate. After planting the bacteria in a bactericidal medium and cultivating for 14 days at 25°, the rinsed,

dried mycelia were weighed and the degree of utilization of organic salts determined. Results of the tests are given in Table 2.

(5) Cellulose Utilization Test. The basic culture medium has the same constituents as in Report 10 (No. II). For cellulose we employed powdered Toyo brand filter paper, and added 1% of it to the basic culture medium. 30 ml each of the prepared culture medium was put into 100 ml Ehrlenmeyer flasks, the bacteria were killed under pressure for 10 minutes at 10 pounds. After the bacteria were planted, they were cultivated for 20 days at 25°. The rinsed and dried mycelia were weighed and the states of growth compared. Results are given in Table 3.

TABLE 2. RESULTS OF TESTS ON UTILIZATION OF ORGANIC SALTS (WEIGHT OF MYCELIA g x 200)

organic salts / strain	Na-Succinate	Na-Fumarate	Na-Citrate	Na-Malonate	Na-Succinate +1/100 M Malonate	Na-Fumarate +1/100 M Malonate	Na-Citrate +1/100 M Malonate
5414	0.20	0.10	0.05	0.00	0.10	0.05	0.05
5418	0.00	0.05	0.05	"	0.05	0.05	0.05
3	0.05	0.05	0.10	"	0.05	0.05	0.10
No. 1	0.45	0.20	0.10	"	0.25	0.20	0.05
No. 2	0.50	0.35	0.05	"	0.10	0.05	0.10
No. 11F8 hetero	0.15	0.15	0.10	"	0.15	0.05	0.10
No. 11 hetero	0.05	0.05	0.10	"	0.05	0.05	0.10
No. 118 hetero	0.10	0.10	0.05	"	0.10	0.05	0.05
P_3	0.10	0.10	0.05	"	0.10	0.05	0.05
A 25	0.05	0.05	0.05	"	0.10	0.10	0.05
A 56	0.20	0.05	0.15	"	0.20	0.05	0.15
5309	0.40	0.05	0.05	"	0.20	0.05	0.05
5311	0.25	0.25	0.05	"	0.30	0.10	0.05
5327	0.60	0.40	0.05	"	0.30	0.30	0.05
5330	0.10	0.05	0.05	"	0.05	0.10	0.05
5333	0.05	0.05	0.10	"	0.10	0.05	0.10
5404	0.05	0.10	0.20	"	0.30	0.05	0.10
5420	0.05	0.05	0.05	"	0.05	0.05	0.05
5424	0.05	0.05	0.05	"	0.05	0.05	0.05
5425	0.05	0.05	0.60	"	0.05	0.02	0.00
5415	0.25	0.20	0.05	"	0.30	0.05	0.05
5514	0.50	0.10	0.10	"	0.50	0.10	0.10
5515	0.15	0.15	0.05	"	0.15	0.15	0.10
5516	0.05	0.05	0.10	"	0.05	0.05	0.05
5517	0.40	0.20	0.10	"	0.25	0.15	0.15
5518	0.05	0.00	0.00	"	0.05	0.00	0.00
5519	0.10	0.05	0.05	"	0.05	0.05	0.05
5520	0.10	0.05	0.00	"	0.05	0.05	0.05
5521	0.05	0.05	0.35	"	0.10	0.02	0.15
5522	0.05	0.05	0.05	"	0.05	0.02	0.05
5523	0.05	0.05	0.10	"	0.05	0.05	0.05
5524	0.10	0.10	0.15	"	0.10	0.10	0.20
5525	0.05	0.05	0.10	"	0.05	0.02	0.05
5526	0.20	0.10	0.05	"	0.20	0.20	0.05
5527	0.15	0.05	0.05	"	0.15	0.05	0.10
5528	0.25	0.05	0.10	"	0.15	0.05	0.15
5529	0.60	0.10	0.05	"	0.70	0.05	0.05
5532	0.25	0.15	0.05	"	0.[illegible]	0.20	0.05
5533	0.05	0.05	0.05	"	0.05	0.05	0.05
5534	0.05	0.05	0.10	"	0.20	0.05	0.10
5535	0.70	0.60	0.05	"	0.60	0.05	0.10
5536	0.05	0.00	0.15	"	0.05	0.05	0.15
5537	0.05	0.05	0.10	"	0.05	0.05	0.05
5539	0.10	0.05	0.05	"	0.05	0.10	0.05
5540	0.05	0.05	0.05	"	0.05	0.05	0.05
CO-homo	0.30	0.05	0.10	"	0.05	0.02	0.10
SO-homo	0.05	0.05	0.05	"	0.05	0.05	0.05

TABLE 3. RESULTS OF CELLULOSE UTILIZATION TESTS.

Strain	Weight of dried mycelia (g)	Strain	Weight of dried mycelia (g)	Strain	Weight of dried mycelia (g)	Strain	Weight of dried mycelia (g)
5414	0.2060	5311	0.0950	5517	0.2200	5529	0.1780
5418	0.2600	5327	0.1430	5518	0.0290	5532	0.1280
3	0.3040	5330	0.3750	5519	0.1480	5533	0.2150
No. 1	0.2080	5333	0.1860	5520	0.1065	5534	0.1970
No. 2	0.2440	5404	0.0900	5521	0.2470	5535	0.3070
No. 11 F8 hetero	0.2675	5420	0.2070	5522	0.1970	5536	0.2290
No. 11 hetero	0.2990	5424	0.1160	5523	0.1400	5537	0.1270
No. 188 hetero	0.3290	5425	0.3520	5524	0.0920	5539	0.1920
P_3	0.2940	5415	0.2100	5525	0.1670	5540	0.2820
A 25	0.3420	5514	0.1780	5526	0.2530	CO-homo	0.1300
A 36	0.2740	5515	0.0970	5527	0.1610	SO-homo	0.1060
5309	0.2000	5516	0.1560	5528	0.3070		

(6) Pectinase test. Conducted according to Abe's method (6). For the test material we used the culture filtrate obtained by cultivating for 14 days at 25° in a medium consisting of the synthetic culture medium (glucose 2%) employed in Report 10 (No. IV) to which 0.5% pectin was added. We prepared the test liquid with the following composition, allowed it to stand at 30° for 24 hours in a beaker, then added 0.5 ml of 1N $CaCl_2$ and tested whether the pectin had hydrolized. Also, for purposes of comparison, we employed a solution in which distilled water was added in place of the test substance.

Constituents of test solution: 2.0 ml of 1% pectin, 2.5 ml of buffer solution (?zerenzen? phosphate pH 5.59), 0.5 ml test substance, 0.1 ml toluol. The results of the above test were negative for pectinase in all strains.

Considerations.

Results of measuring the amount of biotin in the culture filtrate and acid hydrolizate of rinsed mycelia which were cultivated in a biotin-free culture medium (strains 1 and 2 do not require biotin), show that absolutely no biotin was contained in the culture filtrate as shown in Table 1 and in the results in Report 10 (No. IV), while 27.1 - 71.4 mγ/g were contained in the dried mycelia. We established, judging from the weight of the mycelia produced in a biotin-free culture medium, that practically no biotin is produced. This fact shows that biotin is not necessary for development, as was estimated in Report No. 10 (No. II) with regard to strains 1 and 2, and that the type of sugar metabolism differs from other strains.

Using citric acid, succinic acid, and fumaric acid, which participate in the T.C.A. cycle as a carbon source, we studied the role of biotin to see what differences it indicated in the growth of the piricularia oryzae bacteria, by weighing the mycelia. As shown in Table 2, we found that these organic salts and salts to which 1/100 N of malaric acid was added, are not good carbon sources. However, succinic acid shows some influence on growth in strains 1 and 2 from the Agriculture Faculty of Hokkaido University, in strains 5309, 5327, 5514, 5517, 5529, and 5535 of the National Institute of Agricultural Sciences, and in the CO-homo strain from the Agriculture Faculty of the Tokyo University of Agriculture and Technology. Also, strains 2, 5327, and 5535 show some growth in fumaric acid. 5521 shows slight growth in citric acid. No growth was observed in any strains in malaric acid.

An interesting case is that all eight strains, except CO-homo, which grew in succinic acid showed the same rate of growth in media to which 1/100 M malonic acid was added to the succinic acid. Also, of the strains which were reproduced in fumaric acid, Nos. 2 and 5535 did not reproduce at all in cultures to which 1/100 M of malonic acid was added to the fumaric acid, but 5327 showed the same growth as in fumaric acid alone. Recently, various studies have been under way on malonic acid metabolism, and although it cannot be stated definitely (7), we can surmise that the 9 strains above probably undergo a system of metabolism in which the T.C.A. cycle does not participate. Further study of this point is necessary.

The state of growth of piricularia oryzae using powdered filter paper as the carbon source is shown in Table 3. Our classifications of types 1,2,3, and 9 generally show good growth; types 5,7,8, and 11 have poor growth; and no indications were obtained for types 4,6, and 10. Hayami considers that strains which have strong cellulose hydrolizability generally tend toward strong pathogenicity. Such a tendency can also be surmised in general from the information we have obtained, but according to the above results, this tendency cannot serve as a key to classification.

We also conducted tests on pectinase, but with each strain we were unable to detect any noticeable differences.

Recently interesting studies have been made on pectin methylesterase (8) and polygalacturonase (9) and we are intending to test to see whether these might provide a key to classification.

Summary.

Employing 47 strains of piricularia oryzae, we tested the utilization of organic salts involved in the T.C.A. cycle, the utilization of cellulose and the differences in pectinase, as well as the amount of biotin produced by strains 1 and 2 from Hokkaido University, and obtained the following results.

(1) We studied strains 1 and 2, which do not require biotin, by cultivating them in a biotin-free culture medium to see if they are capable of producing biotin. As a result of testing the amount of biotin by bioassay of the acid hydrolizate of rinsed mycelia and of culture filtrate, we did not observe the ability of these strains to produce biotin. These strains are believed to have a different type of sugar metabolism than the other strains.

(2) We tested the utilization of the culture medium containing succinic acid, fumaric acid, and citric acid, and a medium containing the above acids plus 1/100 M of malonic acid. The results lead us to believe that nine strains follow a course different from the T.C.A. cycle.

(3) Although we observed a correlation between utilization and pathogenicity in the tests on the utilization of cellulose, this correlation cannot provide a clue to classification.

(4) We were unable to detect marked differences for pectinase.

(5) We were unable to find a clue to classification from the above results on pectinase and hydrolizability of cellulose.

We would like to express our thanks to professor Hirata Koji of the Pathology Laboratory of the Agriculture Faculty of Niigata University; to assistant professor Iizuka Hiroshi of the Applied Microbiology Laboratory at Tokyo University, who provided us with the Lactobacillus arabinosus; to Professor Suzuki Hashio of the Tokyo University of Agriculture and Technology, who provided us with the homo-types of piricularia oryzae; and to the National Institute of Agricultural Sciences, the Nagano Agricultural test station, the Chugoku agricultural test station, and to the agriculture faculties of Hokkaido and Yamagata universities.

We are also grateful to Mr. Honda Yoshiji of the Education Department of this university for his assistance in the experiments. We wish to acknowledge the Ministry of Education for the Science Research grant, and Mainichi newspaper for the science research scholarship, by means of which our research was made possible.

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